

## **Different gene expression of skin tissues between mice with weight controlled by either calorie restriction or physical exercise**

Jia Lu\*, Linglin Xie\*, Jessica Sylvester, Jiasong Wang, Jianfa Bai\*\*, Richard Baybutt, Weiqun Wang<sup>1</sup>

Department of Human Nutrition, \*\*Gene Expression Facility, Kansas State University, Manhattan, Kansas 66506

\*These authors contributed equally to this work.

<sup>1</sup> To whom correspondence should be addressed at Department of Human Nutrition, Kansas State University, Manhattan, Kansas 66506. E-mail: [wwang@ksu.edu](mailto:wwang@ksu.edu)

<sup>2</sup> Abbreviation used: AL, ad libitum; AE, ad libitum-fed exercise; DCR, dietary calorie restriction; DXA, dual-energy X-ray absorptiometer; GO, gene ontology; PE, pair-fed exercise; TPA, 10-*o*-tetradecanoylphorbol-13 acetate.

## **Abstract**

Cancer prevention by weight control via dietary calorie restriction (DCR) and/or exercise has been demonstrated in animal models. To understand the underlying mechanisms, we compared phorbol ester (TPA)-induced gene expression profiles in DCR- or exercise-treated mouse skin tissues. SENCAR mice were randomly assigned to one of the following four groups: ad libitum-fed sedentary control, ad libitum-fed exercise (AE), exercise but pair-fed at the amount of the control (PE), and 20% DCR. After 10-weeks, both body weight and fat composition significantly decreased in DCR and PE groups when compared with the controls. Weight loss was not observed in AE due to, at least in part, increased food intake. Among 39,000 transcripts with 45,101 probe sets measured by Affymetrix microarray, we identified TPA-induced 411, 110, and 67 genes that showed  $\geq 1.5$ -fold and significant changed by DCR, AE, and PE, respectively. Of these significantly changed genes, gene ontology annotation showed a profound impact on gene expression by DCR in 21 biological process categories. Although PE and AE showed moderate impact on gene expression, the similarity of gene expression pattern altered by PE was relatively closer to DCR, while AE was closer to the control. The results of 22 cancer related gene expression pattern, especially for certain oncogenes, further supported that PE appeared to be a better alternative than AE to DCR-like cancer prevention. The impact on gene expression profile was associated with the effect on weight loss, i.e., DCR  $\gg$  PE  $>$  AE. Overall, this study demonstrated for the first time that weight control via decreasing energy intake or increasing energy expenditure resulted in the different modes of gene expression. Furthermore, DCR showed profound inhibitory impact on the expression of genes relevant to cancer risks.

Furthermore, exercise along with limited dietary calorie intake appears to be a better method for reducing weight and cancer risk when compared to exercise alone.

Key words: weight control, gene expression, calorie restriction, exercise, mice.

## **Introduction**

The National Health and Nutrition Examination Survey in 1999-2000 indicates growing obesity rates in American adults over the past 20 years [1]. Case-control and prospective cohort studies have identified obesity as a risk factor for many types of cancer including colon, breast, endometrial, kidney, and esophageal cancers [2]. High prevalence of overweight and obesity can be explained by a lifestyle characterized with over-consumption of calorie combined with low physical activity [3]. There is ample evidence that weight control via decreasing calorie intake and increasing physical activity reduces cancer risk. International Agency for Research in Cancer of the World Health Organization convened an International Working Group in 2001 and concluded that limiting weight gain during adult life reduces the risk of a number of different types of cancer and increasing physical activity prevents cancer [4].

Studies conducted in animal models indeed demonstrated cancer prevention by weight control via dietary calorie restriction (DCR<sup>2</sup>) or exercise. DCR conducted in lean rodents was found to prevent many types of cancers, including mammary, liver, colon, skin, pancreas, and leukemia [5-10]. Exercise with or without DCR was also reported to have a potential protection against tumor incidence [11-14]. However, it was noted that exercise alone might not consistently result in cancer prevention [15-

17].

Although the mechanisms of DCR and/or exercise for cancer prevention are not clear, some explanations such as alterations of hormone metabolism, hormone-related cellular signaling, oxidation status, DNA repair, apoptosis and oncogene expression, etc. have been postulated [18-20]. Microarray analysis has been applied to cancer prevention experiments by DCR to explore the plausible mechanisms [21-28]. However, relative few data have been published regarding the comparison of gene expression between DCR and exercise, and to date there is no information concerning the gene expression profiles in the combination of dietary calorie intake and energy expenditure for cancer prevention.

To determine the mechanisms underlying such complex relationships between body weight, calorie intake, physical activity, and cancer risk, we applied a genomic microarray analysis to the skin tissues of mice that were controlled in body weight by DCR, exercise, and exercise combined with limited calorie intake. The gene expression profiling was compared and certain phorbol ester-induced cancer-related genes were further exploited.

## **Materials and Methods**

**Animal and Animal Treatment:** Fifty-two female SENCAR mice at 8 weeks of age with body weights averaged at  $30 \pm 2$  g were purchased from NIH (Frederick, MD). Mice were randomly assigned to one of the following four groups: ad libitum-fed sedentary control, ad libitum-fed exercise (AE), exercise but pair-feeding at the amount as ad libitum-fed sedentary counterpart (PE), and 20% DCR. The 20% DCR

diet was formulated by the Harlan Teklad (Madison, WI), containing 20% less total calorie from carbohydrates and fat in comparison with the basal AIN-93 diet, and having extra protein and essential micronutrients to maintain a same level as the basal diet. The amount of the food that each control mouse consumed was recorded weekly and averaged to determine the amount of the following week for DCR and pair-feeding consumption. A zero-grade, motor-driven, adjustable-speed rodent treadmill (Boston Gears, Boston, MA) was used to exercise the mice at 0.5 mph for 60 min per day, 5 days per week for 10 weeks. To take into account the biological clocks of nocturnal mice, we adjusted the light cycle to run nighttime exercise at 0400 to 0500 h. The mice were put on a progressive training program starting at 10% of target exercise duration time and progressing to 100% of the target time by the end of the 2<sup>nd</sup> week. Mice were housed individually in an environmentally controlled room maintained at  $24 \pm 0.6$  °C and 80% relative humidity with a 12 h light/12 h dark cycle starting at 1200 h. Body weights were recorded weekly. Body composition was monitored in the last week by a dual-energy X-ray absorptiometer scan (DXA) using small animal software (v5.6, Prodigy, Lunar-General Electric, Milwaukee, WI). At the end of the experiment when mice were about 20 weeks of age, the dorsal skin of the mice was shaved and topically treated once with TPA at 3.2 nmol. Mice were sacrificed two hours after TPA treatment. The 2-hr period of TPA treatment was selected based on a time course study reported previously by Przybyszewski et al. (29). The dorsal skin samples were snap-frozen in liquid nitrogen and kept at -80 °C until further analyses.

**Microarray Analysis:** Microarray analysis was processed by the Microarray

Core of the Mental Retardation Research Center at the University of Kansas Medical Center (Kansas City, KS). Four mice from each treatment group were used to obtain mouse skin tissues. Each mouse skin tissue (0.4  $\mu$ g) was homogenized in 1 mL TRIZOL reagent and the total RNA was extracted and precipitated by chloroform and isopropanol. Pellet RNA was dissolved in DEPC water and further purified by using the RNeasy cleanup kit (Qiagen, Valencia, CA). The quantity and quality of RNA were measured by using the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). Ten  $\mu$ g of total RNA were then annealed with 100 pmol of T7(dT)<sub>24</sub> at 70 °C for 10 min. The annealed mRNA was reverse transcribed into cDNA using the Superscript Choice System kit (Invitrogen Corp., Carlsbad, CA). Biotinylated antisense cRNA was prepared using the Enzo BioArray High Yield RNA Labeling kit (Enzo Diagnostics, Farmingdale, NY). After purification of labeled cRNA using RNeasy RNA Purification Mini kit (Qiagen, Valencia, CA), 20  $\mu$ g biotin labeled cRNA was incubated in fragmentation buffer (40 mM of Tris-Acetate, 100 mM of potassium acetate, 30 mM of magnesium acetate, pH 8.1) at 94 °C for 35 min. The labeled cRNA then was applied to a GeneChip<sup>R</sup> Mouse Genome 430 2.0 Array (Affymetrix, Santa Clara, CA), containing 39,000 transcripts with 45,101 probe sets. The GeneChip was hybridized, washed, and scanned using Affymetrix equipment and protocols.

**Microarray Data Analysis:** Microarray images were quantified by using GeneChip operating software 1.0 (GCOS 1.0, Affymetrix, Santa Clara, CA). The detection calls of the probe sets were determined using default settings ( $\alpha$ 1, 0.04;  $\alpha$ 2, 0.06;  $\delta$ , 0.015; scale factor, 1.0; norm factor, 1.0). GeneSpring software (Agilent

Technologies, Palo Alto, CA) and Access Database (Microsoft Windows) were used to do data analysis. Scatter plot and condition trees are analyzed by the GeneSpring Software, and distance of gene expression profile were obtained from GeneSpring and compared among groups. Data were first scaled to the same average intensity among all chips to allow fair comparison. Two-step Normalizations were used, including per chip normalization (normalization to 50<sup>th</sup> percentile) and per gene normalization from which the signal intensity in a given chip is divided by the average intensity of the same gene across all chips. According to their expression levels, a discrimination score of a given probe set was calculated based on:  $R = (PM - MM) / (PM + MM)$ , while R is discrimination score, PM is perfect match, and MM is mismatch. The distance between the discrimination score and the given discrimination threshold were tested using One-sided Wilcoxon's Signed Rank test. Probe sets with p-value lower than 0.04 were considered present (P); those with p-value greater than 0.06 were absent (A); and those with p-value in between 0.04 and 0.06 were marginal (M). Furthermore, some microarray data as marked with \_x\_ at suffixes are not unique probe sets or not identical probes among multiple transcripts. Therefore, we excluded all the A, M, and \_x\_ at probe data to increase the data accuracy and reliability. Fold change at 1.5 was used as a cut off to filter experimental data compare with control group. Then ANOVA with Dunnett's' adjustment was applied to assess gene expression difference between the treatment and the control groups. Finally, gene ontology (GO) Slims (<http://www.geneontology.org/GO.slims.shtml>) were applied to classify the differentially expressed genes into 21 GO categories based on the major biological

processes. The categories of TPA-induced cancer related genes including tumor suppressor, apoptosis inducer, apoptosis inhibitor, and oncogene were further presented.

**RT-PCR Confirmation:** RT-PCR reaction was carried out by using purified total RNA obtained as described above. The cDNA was synthesized by RT-PCR using one-step RT-PCR kit (Qiagen, Valencia, CA). The primers are derived from published gene sequences as follows: MAPK1: sense primer 5'-TCT CCC GCA CAA AAA TAA GG-3', antisense primer 5'-TCG TCC AAC TCC ATG TCA AA-3'; H-ras: sense primer 5'-TGT TAC CAA CTG GGA CGA CA-3', antisense primer 5'-TCT CAG CTG TGG TGG TGA AG-3'; PI3Kca: sense primer 5'-TGT TTG CAA AGA AGC TGT GG-3', antisense primer 5'-TAT GAC CCA GAG GGA TTT CG-3'; IGFBP3: sense primer 5'-AAG TTC CAT CCA CTC CAT GC-3', antisense primer 5'-AGC TCT GCT TTC TGC CTT TG-3'; lepr: sense primer 5'-AGG CCC AGA CAT TTT TCC TT-3', antisense primer 5'-TCC TGG AGG ATC CTG ATG TC-3';  $\beta$ -actin: sense primer 5'-TGT TAC CAA CTG GGA CGA CA-3', antisense primer 5'-TCT CAG CTG TGG TGG TGA AG-3'. Fifty  $\mu$ L of PCR reaction were run with a final concentration of 200  $\mu$ M of dNTP mix, 1 x PCR buffer, 1  $\mu$ M of each primer, and 1.0 U of Taq polymerase. Thermal cycling conditions, following an initial denaturation at 94 °C for 4 min, were as follows: 30 sec at 95 °C, annealing at 55 °C for 30 sec, and extension at 72 °C for 1 min. Then samples were incubated at 72 °C for 7 min. Amplified products at 8  $\mu$ L were loaded and separated on a 1.5% agarose gel. The RT -PCR products were visualized under UV light by the FluorChem<sup>TM</sup> 8800 Advanced Imaging System (Alpha Innotech, San Leandro, CA). The relative density of the target band was



normalized to the loading control  $\beta$ -actin and then expressed as a percentage of the controls.

**Statistic Analysis:** The overall effects of treatments on body weight, fat composition, and RT-PCR expression levels were analyzed by one-way ANOVA, and then Dunnett's method and least significant difference (LSD) were used to assess the differences between the treatment groups and the control group. The statistical significance of difference was set at  $P \leq 0.05$ .

## Results

**Impact on body weight and body fat composition:** Lean adult SENCAR mice in the control group gradually gained weight throughout the experimental period, while mice at 20% DCR or exercise with pair-feeding consistently lost weight. By the end of the experiment, the weights of DCR and PE mice were significantly lower than the control mice (Figure 1A). Exercise with ad libitum feeding (AE) did not lower weight significantly when compared with the sedentary counterparts, which might be, at least in part, due to the increased food intake ( $4.0 \pm 0.2$  g/day for the control mice vs.  $4.3 \pm 0.4$  g/day for AE mice). Consequently, percent fat composition, as shown in Figure 1B, significantly decreased in PE and DCR groups but not AE group when compared with the controls. No significant change of bone density was found among groups (data not shown).

**TPA-induced gene expression profiles in response to DCR and exercise treatments:** We measured 45,101 gene probe sets expressed in the skin tissues of the DCR-fed or exercise-treated mice. We compared the distribution of the scatter plots

among AE, PE, and DCR and found that DCR treatment produced the most extensive range of the gene expression levels. A condition tree by using Hierarchical clustering method further displayed the similarity of gene profiles as indicated by a distance between two groups. The smaller the distance is, the closer the gene expression profiling between two groups. Since the results showed a distance at 0.27 between DCR and PE, 0.69 between AE and the control, and 0.96 between DCR and AE, we were convinced that the gene expression pattern of PE group was relatively similar to that of DCR, while AE was closer to the control.

#### **TPA-induced expression of genes regulated by DCR or exercise**

**treatments:** We identified 559 genes that showed at least 1.5-fold significant change ( $P \leq 0.05$ ) by either DCR or exercise treatment when comparing with the control. As shown in Figure 2, we recognized that 411 genes (97 suppressed and 314 over-expressed) were altered by DCR, 110 genes (71 suppressed and 39 over-expressed) were changed by AE, and 67 genes (22 suppressed and 45 over-expressed) were regulated by PE, respectively. It should be noted that two genes relating to RIKEN cDNA (AK009351) and plasmalemma vesical associated protein (NM032398) were down-regulated in both AE and DCR groups, and 6 genes including RIKEN mRNA sequence (BB143476), MARCKS-like protein (NM010807), major urinary protein 3 (M27608), sortilin 1 (AV247637), endothelin receptor B (BB770914), and ATPase (BC001995) were down-regulated in both PE and DCR groups. It was interesting that only one gene corresponding to sequence AA407809 was over-expressed in both AE and PE groups, but 20 genes such as casein kinase II (BG070990), forming binding protein 2 (BB817145), adenylate cyclase 1 (AI848263), sperm associated antigen 1

(NM012031), sialyltransferase 10 (NM018784), SH3 domain protein D19 (NM012059), cystathionine beta-synthase (BC026595), galactosylceramide sulfotransferase (AK002510), and proline dehydrogenase (NM011172) were over-expressed in both PE and DCR groups.

**Functional over-representation analysis:** Functional over-representation analysis by GO annotation was performed to identify major biological processes potentially impacted by the treatments. Figure 3 shows 21 GO categories that were significantly impacted by either calorie restriction or exercise with the percentage of each treatment-induced gene expression frequency. Of the significantly changed genes, DCR showed considerable impact for account of ~ 80% when compared with PE or AE that was usually less than 20%. Specifically, the most genes identified in our study are involved in cell growth/maintenance and cell communication categories, following by DNA binding, transcription factor activity, and transcription categories. A list of all the genes that significantly changed by either DCR or exercise treatment with GO category classification has been provided as a supplementation file.

**RT-PCR confirmation:** the microarray data were further validated by using RT-PCR for five randomly selected genes in cancer-related categories. As shown in Figure 4, five representative gels respectively showed the expression patterns of five genes in comparison with  $\beta$ -actin as a loading control. Given these RT-PCR data, we found the gene expression of MAPK1, PI3Kca and IGFBP3 was significantly decreased in DCR group in comparison with the control group. Both H-ras and lepr were not significantly changed between experimental groups. The RT-PCR

confirmation rate to the microarray data set, as estimated by using a Bayesian statistical method, is about 93.3%.

**Impact on TPA-induced cancer-related genes:** Apart from the genes relevant to nutrient metabolisms and other biological processes, we further identified 22 genes related to TPA-induced cancer risk by using the GO Slims. As shown in Table 1, we found that DCR induced over-expression of 3 tumor suppressor genes, 4 apoptosis inducers, and one apoptosis inhibitor. DCR also induced down-expression of two apoptosis inducers and 8 oncogenes. In comparison with DCR, the impact of PE on expression of these genes is moderate, which induced down-regulation of one apoptosis inducer gene and 4 oncogenes and up-regulation of one apoptosis inducer gene. In contrast, AE was associated with down-expression of one apoptosis inducer gene and one oncogene, but up-expression of one apoptosis inhibitor gene and one oncogene.

## **Discussion**

Weight control can be achieved by either reducing energy intake such as DCR or enhancing energy expenditure like exercise. While 20% DCR reduced body weight significantly, which corresponded to decreased fat composition, the treadmill exercise under this experimental condition demonstrated only a modest weight loss. Exercise alone with ad libitum feeding was not sufficient for decreasing body weight due to, at least in part, the corresponding increase in diet intake. If the food intake of the exercised mice was limited by pair-feeding with their sedentary counterpart, then body weight and fat composition were modestly but significantly reduced. It should

be noted that the interaction of dietary and physical activity upon energy expenditure and homeostasis is complicated. In addition to dietary energy intake and physical activity, for example, the thermogenesis responsible to dietary change and the energy expenditure required for performance of cellular function may also play an interactive role in weight change [30].

By using our established strategies to control body weight in SENCAR mice, we measured the genomic gene expression in TPA-promoted skin tissues and compared the TPA-induced gene expression profiles among AE, PE, and DCR treatments. Of the 39,000 transcripts with 45,101 probe sets measured, we identified 559 genes that showed at least 1.5-fold significant change by DCR and/or exercise treatments in comparison with the controls. The 1.5-fold significant change was selected since it showed a consistent result with a relatively large expression and conservative level [22, 31]. However, the cut off line at 1.5-fold change may exclude genes with <1.5 fold but significantly altered their expression by the treatments.

Of these 559 genes, 411 genes (97 down-expression and 314 over-expression) were altered by DCR, 110 genes (71 down and 39 over) were changed by AE, and 67 genes (22 down and 45 over) were changed by PE, respectively. It is interesting to note the function of some specific genes that were co-regulated significantly by both DCR and PE groups. For example, the gene encoding MARCKS (Myristoylated Alanine-Rich C Kinase Substrate) protein was down-expressed significantly in both PE and DCR groups but not AE group. MARCKS protein is a widely distributed substrate for protein kinase C [32] and activation of protein kinase C has been well recognized as an initial signal in TPA-induced tumor promotion in mouse skin

carcinogenesis [33]. In addition, an up-regulation of histone deacetylase 11 gene (BC016208), one of the large family of sirtunis including SIRT1, by DCR group is in agreement with the published reports by Cohen et al. [34]. Furthermore, SIRT1 has been found to be required for the increased physical activity that is typically observed in calorie restricted mice [35].

When compared with DCR treatment that provides a profound impact on gene expression, the number of gene expression altered by PE and AE are moderate. However, the similarity of gene expression pattern altered by PE and AE appears considerably different. The results by condition tree analysis indicate that the gene expression pattern altered by PE treatment is relatively closer to DCR, while AE is closer to the control. This suggests that moderate exercise alone without diet control may have little effect on DCR-like gene expression pattern. The combination of aerobic exercise with diet control, however, not only lowered body weight but also provided a DCR-like impact on gene expression pattern. This observation is further supported by the results of GO annotation analysis.

By using GO annotation, we identified all the altered genes in 21 GO categories for major biological processes. Specifically, we listed 22 genes that are classified into four TPA-induced cancer-related categories. It is not unexpected that DCR provides an overwhelming impact on the expression of these cancer-related genes, generally in favor of cancer prevention by inducing over-expression of tumor suppressor and apoptosis inducer genes as well as down-expression of oncogenes. It is interesting that PE induced a moderate, but similar impact as DCR on oncogene expression, especially in down-regulating the oncogenes. In contrast, AE showed the

least impact on these cancer related genes. Considering the impact on body weight and fat composition as well as the similarity of gene expression pattern, we may deduce that PE could be a better alternative than AE to DCR-like cancer prevention via modifying gene expression pattern such as down-regulation of certain oncogenes.

Frankly speaking, it is challenging and ambitious to specify all the significantly changed genes and their potential roles accounted for cancer prevention. Although microarray analysis is useful for identifying potential gene expression and enhance our understanding of the cancer prevention by weight control, future studies by incorporating gene expression data with proteomics may provide more insights.

Nevertheless, this pilot study reports the altered mRNA expression of certain genes in weight control mice via either reduced dietary energy intake or increased energy expenditure. DCR treatment provided substantial weight loss and significantly modified the gene expression profile. PE induced a modest impact on both weight loss and gene expression. In contrast to PE, AE was not associated with reduced body weight and its effect on gene expression pattern was more similar to the controls. The data suggest that the degree of weight loss may be the critical indicator in reducing cancer risks.

### **Acknowledgements**

We acknowledge the DXA analysis assistance of Dr. Mark Haub (Department of Human Nutrition, Kansas State University), the computational advice of Dr. Kesheng Liu (Department of Plant Pathology, Kansas State University), and the assistance in microarray data analysis from Mrs. Nanyan Weng (Gene Expression

Facility, Kansas State University). This study is supported by an Innovative Research Grant from the Terry Johnson Center for Basic Cancer Research, Kansas State University, grant COBRE P20 RR15563, and grant R01 CA106397 from the National Institute of Health. This is a journal contribution # 04-429-J of the Kansas Agriculture Experiment Station, Manhattan, Kansas.



## References:

1. Flegal KM, Carroll MD, Ogden CL, Johnson CL. Prevalence and trends in obesity among US adults, 1999-2000. *J Am Med Assoc* 288:1723-1727, 2002.
2. Bianchini F, Kaaks R, Vainio H. Overweight, obesity, and cancer risk. *Lancet Oncol* 3:565-574, 2002.
3. Scott MG. Multifactorial causation of obesity: implications for prevention. *Am J Clin Nutr* 67: 563S-572S, 1998.
4. Vainio H, Kaaks R, Bianchini F. Weight control and physical activity in cancer prevention: international evaluation of the evidence. *Eur J Cancer Prev* 2:S94-100, 2002.
5. Kritchevsky D. Caloric restriction and experimental mammary carcinogenesis. *Breast Cancer Res Treat* 46:161-167, 1997.
6. Kolaja KL, Bunting KA, Klaunig JE. Inhibition of tumor promotion and hepatocellular growth by dietary restriction in mice. *Carcinogenesis* 17:1657-1664, 1996.
7. Pollard M, Luckert PH, Pan GY. Inhibition of intestinal tumorigenesis in methylazoxymethanol-treated rats by dietary restriction. *Cancer Treat Rep* 68:405-408, 1984.
8. Birt DF, Pinch HJ, Barnett T, Phan A, Dimitroff K. Inhibition of skin tumor promotion by restriction of fat and carbohydrate calories in SENCAR mice. *Cancer Res* 53:27-31, 1993.
9. Robuck BD, Yager JD, Longnecker DS. Dietary modulation of azaserine-induced pancreatic carcinogenesis in the rat. *Cancer Res* 41:888-93, 1981.
10. Yoshida K, Inoue T, Nojima K, Hirabayashi Y, Sado T. Calorie restriction reduces the incidence of myeloid leukemia induced by a single whole-body radiation in C3H/He mice.

- Proc Natl Acad Sci USA 94:2615-2619, 1997.
11. Kritchevsky D. Influence of caloric restriction and exercise on tumorigenesis in rats. Proc Soc Exp bio Med 193:35-38, 1990.
  12. Davis JM, Kohut ML, Jackson DA, Colbert LH, Mayer EP, Ghaffar A. Exercise effects on lung tumor metastases and in vitro alveolar macrophage antitumor cytotoxicity. Am J Physiol 274:R1454-1459, 1998.
  13. Hoffman-Goetz L, May KM, Arumugam Y. Exercise training and mouse mammary tumor metastasis. Anticancer Res 14:2627-2631, 1994.
  14. Lane HW, Teer P, Keith RE, White MT, Strahan S. Reduced energy intake and moderate exercise reduce mammary tumor incidence in virgin female BALB/c mice treated with 7,12-dimethylbenz( $\alpha$ ) anthracene. J Nutr 121:1883-1888, 1991.
  15. Thompson HJ, Ronan AM, Ritacco KA, Tagliaferro AR, Meeker LD. Effect of exercise on the induction of mammary carcinogenesis. Cancer Res 48:2720-2723, 1988.
  16. Thompson HJ, Ronan AM, Ritacco KA, Tagliaferro AR. Effect of type and amount of dietary fat on the enhancement of rat mammary tumorigenesis by exercise. Cancer Res 49: 1904-1908, 1989.
  17. Thompson HJ, Westerlind K, Snedden JR, Briggs S, Singh M. Inhibition of mammary carcinogenesis by treadmill exercise. J Natl Cancer Inst 87: 453-455, 1995.
  18. Kritchevsky D. Caloric restriction and experimental carcinogenesis. Hybrid Hybridomics 21:147-151, 2002.
  19. Zhu Z, Jiang W, Thompson HJ. Mechanism by which energy restriction inhibits rat mammary carcinogenesis: in vivo effects of corticosterone on cell cycle machinery in mammary carcinomas. Carcinogenesis 24:1225-1231, 2003.

20. Rogers AE, Zeisel SH, Groopman J. Diet and carcinogenesis. *Carcinogenesis* 14:2205-2217, 1993.
21. Cao SX, Dhahbi JM, Mote PL, Spindler SR. Genomic profiling of short- and long- term caloric restriction effects in the liver of aging mice. *Proc Natl Acad Sci USA* 98:10630-10635, 2001.
22. Dhahbi JM, Kim HJ, Mote PL, Beaver RJ, Spindler SR. Temporal linkage between the phenotypic and genomic responses to caloric restriction. *Proc Natl Acad Sci USA* 101: 5524-5529, 2004.
23. Lee CK, Klopp RG, Weindruch R, Prolla TA. Gene expression profile of aging and its retardation by caloric restriction. *Science* 285:1390-1393, 1999.
24. Jevic KM, Robaire B. Effects of caloric restriction on gene expression along the epididymis of the Brown Norway rat during aging. *Exp Gerontol* 38:549-60, 2003.
25. Tsuchiya T, Dhahbi JM, Cui X, Mote PL, Bartke A, Spindler SR. Additive regulation of hepatic gene expression by dwarfism and caloric restriction. *Physiological Genomics* 17: 307-315, 2004.
26. Higami Y, Barger JL, Page GP, Allison DB, Smith SR, Prolla TA, Weindruch R. Energy restriction lowers the expression of genes linked to inflammation, the cytoskeleton, the extracellular matrix, and angiogenesis in mouse adipose tissue. *J Nutr* 136:343-352, 2006.
27. Sreekumar R, Unnikrishnan J, Fu A, Nygren J, Short KR, Schimke J, Barazzoni R, Nair KS. Effects of caloric restriction on mitochondrial function and gene transcripts in rat muscle. *Am J Physiol Endocrinol Metab* 283:E38-43, 2002.
28. Han ES, Hickey M. Microarray evaluation of dietary restriction. *J Nutr* 135:1343-1346, 2005.

29. Przybyszewski J, Yaktine AL, Duysen E, Blackwood D, Wang W, Au A, Birt DF. Inhibition of phorbol ester-induced AP-1-DNA binding, c-Jun protein and c-jun mRNA by dietary energy restriction is reversed by adrenalectomy in SENCAR mouse epidermis. *Carcinogenesis* 22:1421-1427, 2001.
30. Badman MK, Flier JS. The gut and energy balance: visceral allies in the obesity wars. *Science* 307:1909-1914, 2005.
31. Petersen D, Chandramouli GV, Geoghegan J, Hilburn J, Paarlberg J, Kim CH, Munroe D, Gangi L, Han J, Puri R, Staudt L, Weinstein J, Barrett JC, Green J, Kawasaki ES. Three microarray platforms: an analysis of their concordance in profiling gene expression. *BMC Genomics* 6:63, 2005.
32. Stumpo DJ, Eddy RL Jr, Haley LL, Sait S, Shows TB, Lai WS, Young WS 3rd, Speer MC, Dehejia A, Polymeropoulos M, Blackshear PJ. Promoter sequence, expression, and fine chromosomal mapping of the human gene (MLP) encoding the MARCKS-like protein: identification of neighboring and linked polymorphic loci for MLP and MACS and use in the evaluation of human neural tube defects. *Genomics* 49:253-264, 1998.
33. Wheeler DL, Ness KJ, Oberley TD, Verma AK. Protein kinase Cepsilon is linked to 12-O-tetradecanoylphorbol-13-acetate-induced tumor necrosis factor-alpha ectodomain shedding and the development of metastatic squamous cell carcinoma in protein kinase Cepsilon transgenic mice. *Cancer Res* 63:6547-6555, 2003.
34. Cohen HY, Miller C, Bitterman KJ, Wall NR, Hekking B, Kessler B, Howitz KT, Gorospe M, de Cabo R, Sinclair DA. Calorie restriction promotes mammalian cell survival by inducing the SIRT1 deacetylase. *Science* 305:390-392, 2004.
35. Chen D, Steele AD, Lindquist S, Guarente L. Increase in activity during calorie

restriction requires Sirt1. Science 310:1641, 2005.

## Figure legends:

### **Figure 1: Effects of DCR and exercise-treatment on body weight and fat composition.**

A: body weight; B: fat composition. Results are means  $\pm$  SE, n=8-12. Means with different alphabetical letters differ significantly,  $p \leq 0.05$ .

### **Figure 2: Venn diagram with number of genes altered in each group, shared across two**

**groups, and shared across all three groups.** Left: Genes that are 1.5-fold significantly down-expressed in comparison with the control, Right: Genes that are 1.5-fold significantly over-expressed in comparison with the control.

### **Figure 3: Functional over-representation analysis of gene expression altered by DCR and**

**exercise treatments.** Gene ontology Slims were used to show the percentage of TPA-induced gene expression altered by AE, PE, and DCR, respectively, with a given gene ontology category. Each gene category shown is the combined genes that are significantly altered ( $p < 0.05$ ) at greater than 1.5 fold change. The number of genes for each category is shown in parenthesis.

### **Figure 4: Confirmation of microarray data by RT-PCR.**

Five genes were randomly chosen from cancer-related categories, and their expression pattern in comparison with the microarray data was validated by RT-PCR. Identical results were obtained with MAPK1, PI3Kca, and IGFBP3 genes that down-expressed significantly in DCR group. The expression of  $\beta$ -actin gene was used as a loading control.

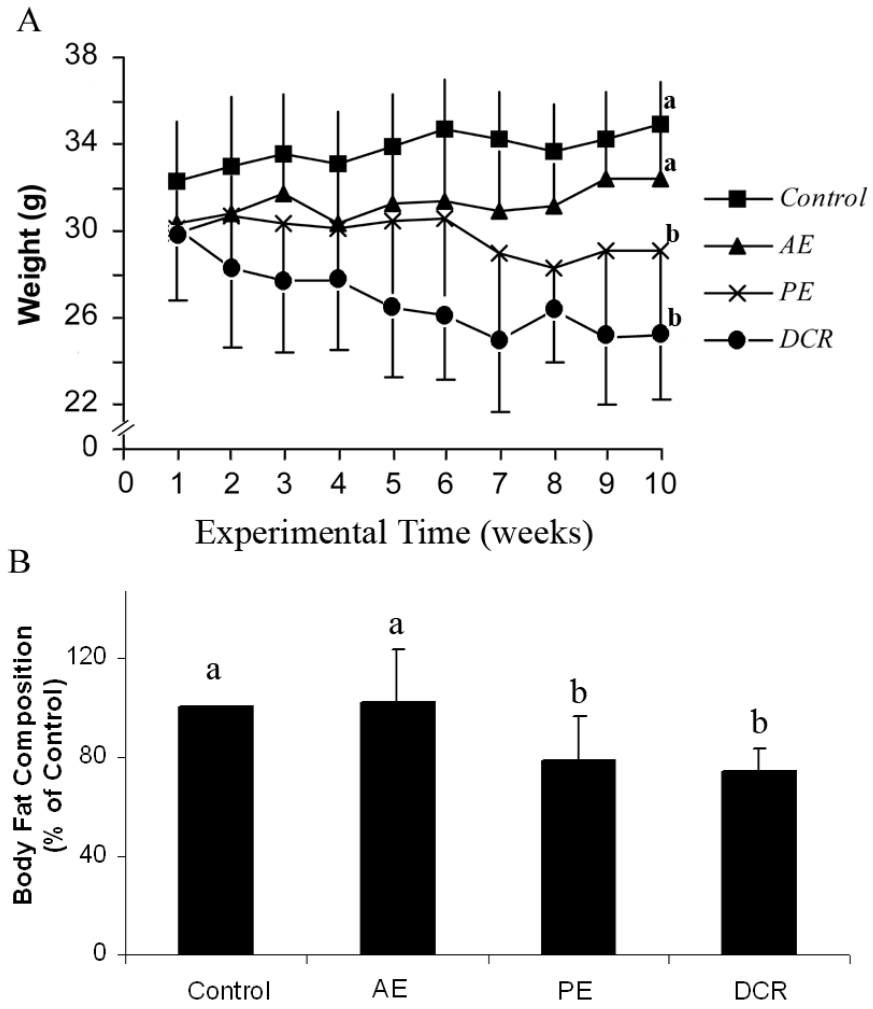


Figure 1

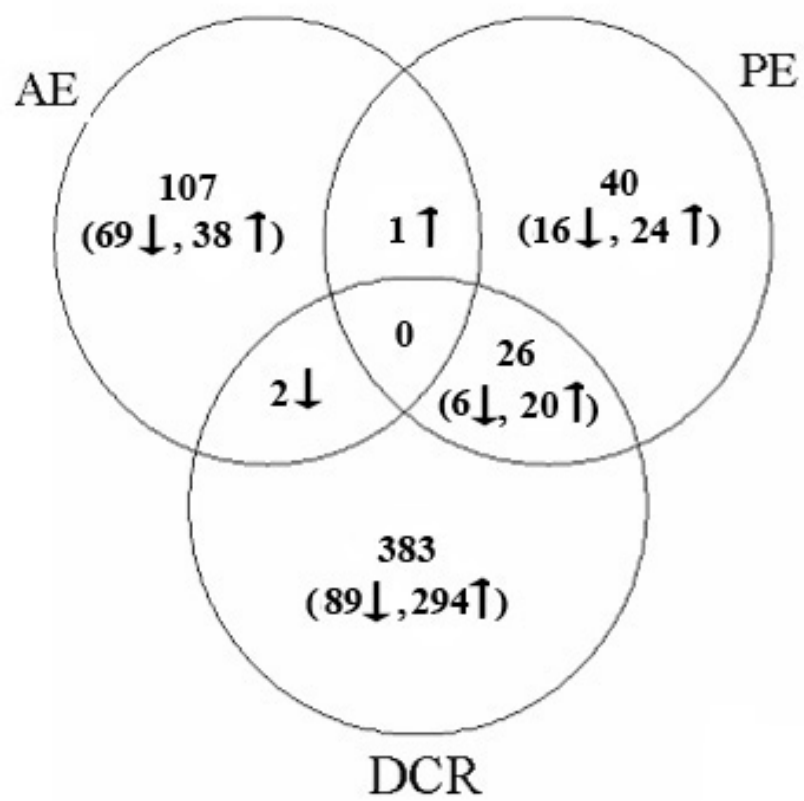


Figure 2



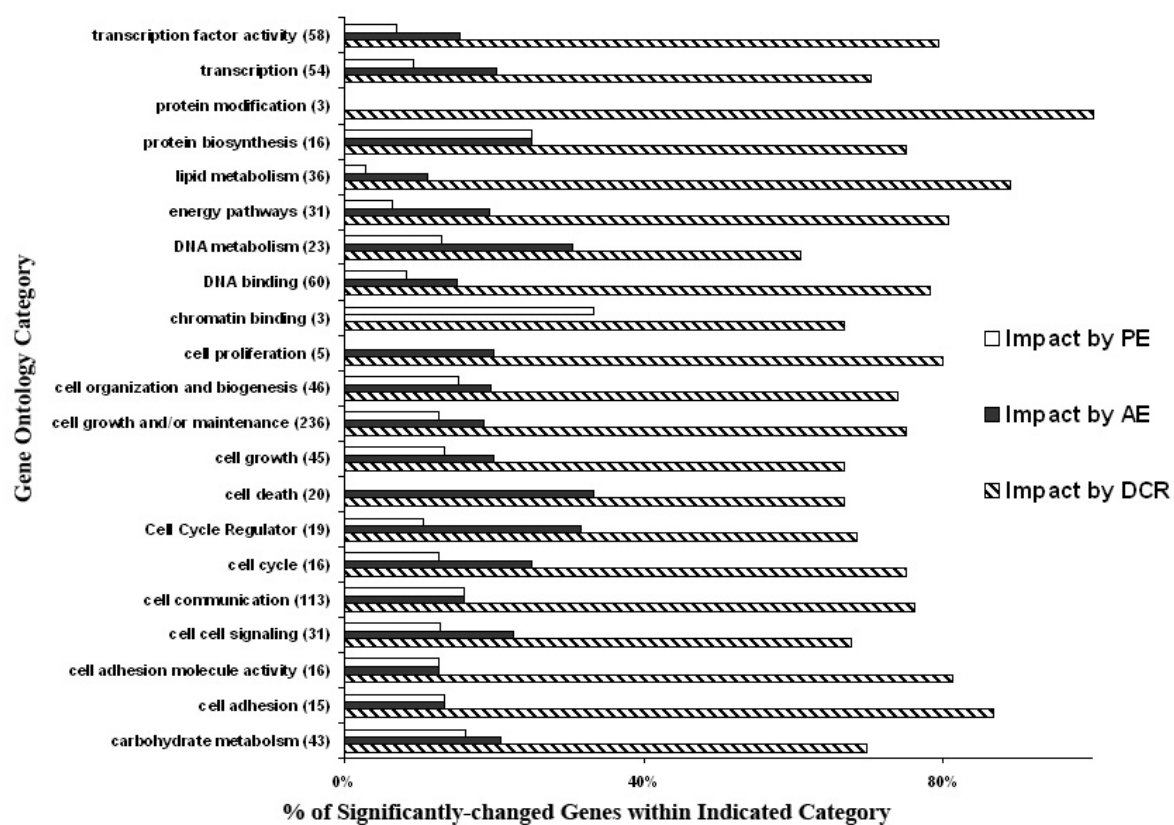


Figure 3

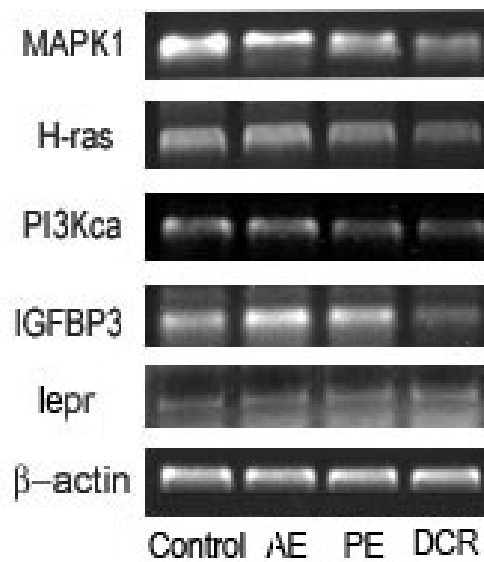


Figure 4

**Table 1. Identified phorbol ester-induced cancer related genes altered by DCR, PE, and AE treatments, respectively.**

Ontology Category	Probe	Gene Name	Fold Change (p value)*		
			DCR	PE	AE
<b>Tumor Suppressor</b>	1429418_at	RIKEN cDNA A530086E13 gene	+1.52 (p<0.036)		
	1418146_a_at	retinoblastoma-like 2	+1.61 (p<0.030)		
	1454867_at	Mus musculus transcribed sequence with moderate similarity to protein sp:Q10571 (H.sapiens) MN1_HUMAN Probable tumor suppressor protein MN1	+1.70 (p<0.024)		
<b>Apoptosis Inducer</b>	1449317_at	CASP8 and FADD-like apoptosis regulator	-2.91 (p<0.002)	-1.62 (p<0.002)	
	1418011_a_at	SH3-domain GRB2-like B1 (endophilin)	+1.52 (p<0.029)		
	1450731_s_at	tumor necrosis factor receptor superfamily, member 21	+1.96 (p<0.026)	+1.80 (p<0.026)	
	1433938_at	transformation related protein 53 binding protein 2	+1.72 (p<0.025)		
	1449591_at	caspase 4, apoptosis-related cysteine protease	+1.59 (p<0.014)		-1.81 (p<0.045)
	1426184_a_at	Programmed cell death 6 interacting protein	-2.12 (p<0.042)		
<b>Apoptosis Inhibitor</b>	1449033_at	tumor necrosis factor receptor superfamily, member 11b (osteoprotegerin)	+2.31 (p<0.031)	+1.98 (p<0.031)	
	1435024_at	nucleolar protein 3 (apoptosis repressor with CARD domain)			+1.58 (p<0.035)
<b>Oncogene</b>	1422687_at	neuroblastoma ras oncogene	-1.98 (p<0.034)		
	1451715_at	v-maf musculoaponeurotic fibrosarcoma oncogene family, protein B (avian)		-1.64 (p<0.008)	
	1427783_at	v-erb-a erythroblastic leukemia viral oncogene homolog 4 (avian)	-3.34 (p<0.033)	-2.47 (p<0.033)	-1.69 (p<0.033)
	1417155_at	neuroblastoma myc-related oncogene 1	-3.31 (p<0.008)	-1.93 (p,0.008)	
	1424332_at	Rab40c, member RAS oncogene family	-2.64 (p<0.010)		
	1417656_at	myeloblastosis oncogene-like 2	-2.18 (p<0.001)	-1.64 (p<0.001)	
	1422087_at	lung carcinoma myc related oncogene 1	-2.02 (p<0.007)		
	1450194_a_at	myeloblastosis oncogene	-1.95 (p<0.040)		+1.67 (p<0.040)
	1422119_at	RAB5B, member RAS oncogene family	-1.88 (p<0.035)		
	1448885_at	RAP2B, member of RAS oncogene family	-1.70 (p<0.006)		
	1416591_at	RAB34, member of RAS oncogene family	-1.52 (p<0.035)		

\* The fold change is a ratio of the gene expression in the treatment group to the controls, which is denoted as increased (+) or decreased (-) if the treatment group is greater or less than the

control, respectively. Only the data that are greater or less than 1.5-fold with a significant change ( $p < 0.05$ ) are present ( $n = 4$  per group).